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Photosynthetic response of four fern species from different habitats to drought stress: relationship between morpho-anatomical and physiological traits

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Abstract

Ferns flourish in many habitats, from epiphytic to terrestrial and from sunny to shady, and such varied conditions require contrasting photosynthetic strategies to cope with drought. Four species of temperate ferns from different habitats were subjected to drought by withholding irrigation in order to investigate their photosynthetic responses. *Lepisorus thunbergianus* (epiphytic) had low stomatal density and showed high water-use efficiency (WUE) retaining photosynthetic activity with low relative frond water content under drought stress, which suggested their high adaptation to drought. On the other hand, low WUE with low light-saturated photosynthetic rate in *Adiantum pedatum* (terrestrial, shady environment) was associated with much lower photosynthesis than in the other species under drought stress, suggesting lower adaptation to drought-prone habitats. Morphological stomatal traits such as stomatal density and photosynthetic response to drought in ferns involved species-specific adaptation to survive and grow in their natural habitats with different levels of drought.

Additional key words: gas exchange; Japan; pteridophyte; stomatal size; water stress.

Introduction

Drought stress is one of the most important factors determining the growth and survival of higher plants, in which the leaf photosynthetic response is a fundamental process affected by drought (Chaves *et al.* 2009). The effect of drought stress on photosynthesis is even more critical for ferns, because the fronds of ferns exhibit less resistant xylem to embolism compared to angiosperms (Woodhouse and Nobel 1982, Brodribb and Holbrook 2004). Ferns are an important component of temperate forests as well as tropical rainforests and have significant roles in tree regeneration and forest biodiversity (Royo and Carson 2006), accounting for more than 10,000 species worldwide (Pryer *et al*. 2004, Smith *et al*. 2006, http://www.theplantlist.org/1.1/browse/P/). Although many fern species prefer wet and shady habitats, such as the forest understory, where drought is less frequent, ferns with epiphytic life-forms, which dominate the epiphytic community in temperate forests (Zotz 2005), are more likely to be exposed to drought compared to terrestrial ferns (Watkins *et al.* 2007). Some ferns growing in sunny habitats, where high light levels for photosynthesis are available, may also experience more severe drought compared to those in shady habitats (Saldanã *et al.* 2005). Therefore, ferns with different life-forms or habitats, *e.g.*, epiphytic or terrestrial and sunny or shady, should exhibit contrasting photosynthetic strategies in order to cope with drought. However, understanding of the photosynthetic responses to drought in fern species in relation to their life-forms and habitats is limited compared to that on seed plants.

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Abbreviations: C_i – internal CO_2 concentration; DM – dry mass of frond; FM – fresh mass of frond; FWC – frond water content; g_s – stomatal conductance; J – electron transport rate; LMA – leaf mass per area; P_{max} – light-saturated photosynthetic rate; P_N – net photosynthetic rate; WUE (P_{max}/g_s) – photosynthetic water-use efficiency; RFWC – relative frond water content; SWC – relative soil water contents; TM – turgid mass of frond; V_{cmax} – maximum carboxylation rate; VPD – vapor pressure deficit; $\delta^{13}C$ – carbon isotope ratio.

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In seed plants, an increase in photosynthetic water-use efficiency (WUE) occurs as an important adaptive process to drought unless the degree of drought is less severe (Flexas *et al.* 2004, Galmés *et al.* 2007). Their response is strongly affected by the plant life-form (Medrano *et al.* 2009). Therefore, it is highly possible that differences in life-form or habitat also cause different responses in photosynthesis (P_{max}) and stomatal conductance (g_s) to drought among fern species. Although studies of the WUE response (P_{max}/g_s) to drought in ferns are limited, some previous studies reported increases in instantaneous and long-term WUE in a fern *Pteridium aquilinum* in response to drought (Gordon *et al.* 1999), and epiphytic ferns showed higher P_{max}/g_s in response to drought compared to terrestrial ferns (Zhang *et al.* 2009).

The decrease in g_s as well as the changes in P_{max} may involve species-specific responses of WUE in fern species, because in seed plants, both low g_s and more suited photosynthetic metabolism to drought conditions contribute to the increase in P_{max}/g_s in response to drought (Flexas and Medrano 2002). The g_s of fern species operates in a different way than in seed plants; the fern *Adiantum capillus-veneris* and other eusporangiate ferns lack g_s responses to blue light or CO_2 in the dark (Doi *et al.* 2006, 2015, Doi and Shimazaki 2008). Some fern species showed small changes in g_s in response to an increase in atmospheric CO_2 (Brodribb *et al.* 2009, Brodribb and McAdam 2013, Nishida *et al.* 2015). The mechanisms relating stomatal response to drought are also different between seed plants and ferns; stomatal responses of ferns are passively driven by changes in leaf water status, whereas the stomata of seed plants are actively regulated by abscisic acid (Brodribb and McAdam 2011). Irrespective of such different regulatory mechanisms of stomata, ferns showed declines in g_s in response to drought (*e.g.* Prange *et al.* 1983, Zhang *et al.* 2009, McAdam and Brodribb 2013), which were similar to seed plants. However, the response of photosynthetic biochemistry to drought remains to be clarified for ferns.

Morphological stomatal traits, such as length, width, and frequency, vary between genotypes in seed plants (Hetherington and Woodward 2003) and have fundamental roles in determining the response of g_s to drought. Stomatal length and width are fixed genetically at a species level in *Blechnum* ferns (Galán *et al.* 2011), whereas stomata frequency varies much more strongly within than between species, in response to environmental changes (Kessler *et al.* 2007). Contradictory results have been reported on the relationship between stomatal density and g_s in response to environmental changes in seed plants; higher stomatal density has been usually reported to relate to higher sensitivity of stomata to environmental changes (Woodward 1987), whereas lower stomatal density was related to higher sensitivity of stomata to drought in Mediterranean plants (Medrano *et al.* 2009). To the best of our knowledge, there have been no studies analyzing the drought response of ferns in relation to morphological stomatal traits, and such a study could provide valuable information for understanding the physiological mechanisms of drought response in ferns.

We hypothesized that different life-forms or habitats of ferns involve contrasting photosynthetic strategies to cope with drought, in which morphological stomatal traits could have a significant impact. We expected that epiphytic ferns, evergreen ferns, and those from sunny habitats would show better adaptation to drought compared to terrestrial ferns, deciduous ferns, and those from shady habitats, respectively. We focused on the Japanese temperate ferns *Adiantum pedatum*, *Pteridium aquilinum*, *Dryopteris erythrosora*, and *Lepisorus thunbergianus*. They have different life-forms and habitats; *A. pedatum* is a terrestrial, deciduous species that grows in shady moist habitats. *P. aquilinum* is a terrestrial, deciduous species that grows in sunny habitats such as open forests or grasslands. *D. erythrosora* is a terrestrial, evergreen species that grows in forest borders or understory habitats, while *L. thunbergianus* is an epiphytic, evergreen species. These four species were subjected to drought stress by withholding irrigation in order to investigate their photosynthetic responses. The aim of this study was to characterize the responses of frond photosynthesis to drought and to determine if such characteristics are related to the habitat adaptation of each species. Our second aim was to identify the effect of species-specific frond morphological, stomatal, and biochemical traits of photosynthetic responses to drought.

Materials and methods

Plant material: Four fern species with different life-forms and habitats were used in the present study (Fig. 1). *A. pedatum* L., *P. aquilinum* (L.) Kuhn var. *latiusculum* (Desv.) Underw., and *D. erythrosora* (Eat.) O.Ktze. were obtained commercially (*Takayama-engei*, Kyoto, Japan), whereas *L. thunbergianus* (Kaulf.) Ching was collected from around the campus of Kyoto Institute of Technology, Japan. Ten to twelve plants for each species were grown in a glasshouse with a 50% shading cloth, where the average daily PPFD was 500 μmol m^{-2} s^{-1}. *A. pedatum* and *D. erythrosora* were planted in 1.2-L Wagner pots, whereas *P. aquilinum* and *L. thunbergianus* were planted in 1.0-L and 0.3-L vinyl pots, respectively. The pots were filled with peat moss, leaf mold, and sand mixed at a mass ratio of 3:3:1. Plants were irrigated every 1 or 2 d and fertilized once a week with a 1/1,000 nutrient solution (N:P:K = 5:10:5, *Hyponex*, USA) before drought treatment was started.

Leaf mass per area and stomatal traits: In 2010, ten fully expanded mature fronds were collected from different individuals per species before the drought treatment was started. Frond area was determined using a scanner (*GT7600U*, *Epson*, Nagano, Japan) and analyzed with *ImageJ* software (*ImageJ 1.47*, U. S. National Institutes of Health, Bethesda, Maryland, USA). Thereafter, fronds were dried at 70°C for more than 48 h until their mass was constant, and then their dry masses were measured to determine leaf mass per area (LMA).

For the analysis of stomatal traits, impressions of the epidermis were obtained from a fully expanded mature frond collected from four plants per species using silicon rubber and clear nail polish. Stomata density was measured by counting the number of stomata in the impressions using a microscope (*BX51*, *Olympus*, Tokyo, Japan) at ×100 magnification. Stomata length and width were measured for five stomata from each impression ($n = 20$) using a light microscope at ×200 magnification.

Drought treatment was performed from June to November in 2010 and 2011. The mass of each pot ($n = 10$ for each species) was measured every 1 or 2 d during the treatment period. Relative soil water contents (SWC) was calculated using the following equation:

SWC [%] = (remaining water/maximum amount of water) × 100 (1)

where remaining water = maximum amount of water – water loss. The maximum amount of water available per pot was obtained by subtracting the dried-soil mass from the fully irrigated-soil mass. Fully irrigated-soil mass was determined when excess of water was drained from the pot following irrigation. For the control treatment, 4–5 plants for each species were irrigated every 2 or 3 d to keep the SWC at more than 80% for all species. For the drought treatment, 4–5 plants for each species were used, with irrigation stopped when SWC reached 40–50%. For *A. pedatum*, *D. erythrosora*, and *P. pedatum*, irrigation then started again to keep SWC at 20–40%. For *L. thunbergianus*, SWC decreased so slowly that irrigation was not performed during the drought treatment. Drought treatment was finished when g_s decreased to 20% of the species' maximum g_s.

Frond gas exchange and water content: Gas exchange from a fully expanded mature frond with no visible damage was measured 3–5 times for each species; at the beginning of the treatment, on days when relative SWC reached 40–50%, and thereafter, every week until the drought treatments were finished. The measurements were conducted from 9 to 17 h. Frond gas-exchange measurements were performed using a *Li-6400* photosynthesis system (*Li-Cor Inc.*, Nebraska, USA) at a frond temperature of 25 ± 0.5°C and a vapor pressure deficit (VPD) of 1.0 ± 0.1 kPa. P_{max} and g_s were obtained with 400 μmol mol^{-1} CO$_2$ and saturating PPFD of 1500 μmol m^{-2} s^{-1} for *P. aquilinum* and 500 μmol m^{-2} s^{-1} for the other ferns after 10-min acclimation. P_N/C_i curves were obtained under light-saturated PPFD conditions. CO$_2$ concentration was first set at 400 μmol mol^{-1}, and then decreased to 300, 200, 100, and 50 μmol mol^{-1}, and thereafter, increased to 400, 500, 600, 700, 1,000, 1,500, and 2,000 μmol mol^{-1}. Fronds were acclimated for at least 5 min at each step. Maximum carboxylation rate (V_{cmax}) and electron transport rate (J) were calculated using the P_N/C_i curve fitting method (*A*/*C*$_i$ *Curve Fitting 10.0.xls*, http://landflux.org/Tools.php, Ethier and Livingstone 2004). After gas-exchange measurements, the frond was cut and scanned using a scanner (*CanoScan 9930 F*, *Canon*, Tokyo, Japan). Leaf area was measured from the scanned images using *ImageJ* software (http://imagej.nih.gov/ij/).

To investigate the effect of drought on water relations in fronds and compare them between species, frond water content (FWC) and relative frond water content (RFWC) were calculated using the following equations:

FWC [%] = (FM – DM) × 100/ DM (2)

RFWC [%] = (FM – DM) × 100/(TM – DM) (3)

where FM, DM, and TM are fresh mass (FM) at the time of sampling, dry mass (DM) after drying for more than 48 h at 70°C, and turgid mass (TM) of fronds, respectively. TM was measured after 2 d of soaking the fronds in distilled water.

Rubisco content in fronds: The Rubisco content was measured in 2011 in accordance with the method of Kawase *et al.* (2013) with some modifications. The fronds used for gas-exchange measurements were stored at −80°C and then ground in liquid nitrogen. They were homogenized in 1% polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride, 4% lithium dodecyl sulfate, and 600 μl of Rubisco extraction buffer (pH 7.5) containing 50 mM HEPES, 0.2% Triton X-100, 0.7% 2-mercaptoethanol, 2 mM 2-iodoacetic acid, and 25% glycerol (Kursar and Coley 1992). The supernatants were subjected to SDS-PAGE [12.5% gels, 4 μm loaded, stained with *Coomassie Brilliant Blue G-250* (*Bio-safe Coomassie*, *Bio-Rad*, USA)], and Rubisco contents were analyzed bz creating a standard curve in the same gel using a scanner (*GT-7600U*, *Epson*, Nagano, Japan) and *Densitograph* software (*ATTO,* Tokyo, Japan).

Carbon isotope ratio: Fully expanded mature fronds with no visible damage were collected at the end of the drought treatment in 2010. The fronds were completely dried in a drying furnace at 60°C for 2 d and then ground into fine powder using an agate mortar. Subsamples of 1–2 mg were subjected to isotope measurements using a combined system of an elemental analyzer (*EA1108, Carlo-Erba*, Italy), an interface (*Finnigan MAT ConfloII*, Bremen, Germany), and an isotope mass spectrometer (*Finnigan MAT Delta S*, Bremen, Germany). The isotope values were corrected using standards (Tayasu *et al.* 2011).

Statistical analysis was performed using *EZR* software (*EZR version 1.24*, Kanda 2013). Frond traits, photosynthetic traits, and carbon isotope ratio between species were tested using analysis of variance (*ANOVA*) and *Tukey*'s test. For drought treatments, parameters between the control and the drought conditions were tested using *t*-test.

Results

Stomatal traits, frond water content and leaf mass per area: Stomata were present only on the abaxial side of fronds in all ferns (Fig. 1). The two evergreen ferns, *D. erythrosora* and *L. thunbergianus*, showed longer stomatal length than the two deciduous ferns, *A. pedatum* and *P. aquilinum* (Table 1). *L. thunbergianus* showed wider stomatal width than that of the other three ferns. Stomatal density of *P. aquilinum* was more than twice that of the other three ferns. FWC was the highest for the epiphyte fern *L. thunbergianus*. The evergreen ferns, *D. erythrosora* and *L. thunbergianus*, had significantly higher LMA than that of the deciduous ferns, *A. pedatum* and *P. aquilinum*.

Responses of frond gas exchange to drought: The data obtained in 2010 and 2011 were pooled, because of a similar plant size, and there were no significant differences in g_s for the control plants between 2010 and 2011 before the onset of drought treatment (data not shown). Average relative soil water content (SWC) for drought-treated and control plants was 27.1–36.0%, and 88.3–91.2%, respectively, with no significant difference between species for both control and drought-treated plants.

In the last week of drought treatment, g_s for the drought-treated plants declined to 21–50% of the values of the control plants (Table 2). In this study, g_s was classified into three ranges, <30%, 30–60%, and >60% of maximum g_s for each plant species, to assess the effect of drought on photosynthesis in the fronds (Flexas and Medrano 2002). Lower g_s ranges indicated a higher degree of drought stress.

Increasing intrinsic WUE (P_{max}/g_s) is an adaptive trait for gas exchange in response to drought stress. When P_{max}/g_s values were compared between g_s ranges, all fern species showed their highest values at g_s of <30% (Fig. 2). When WUE values were compared between the species, *D. erythrosora* and *L. thunbergianus* showed higher values than the other species when g_s was <30% and 30–60%. The carbon isotope ratio ($\delta^{13}C$) of frond DM can also be used to estimate long-term frond water-use efficiency (Hanba *et al.* 2003). However, drought stress did not affect $\delta^{13}C$ in the fronds of any of the species examined (Table 2). When comparing species, *D. erythrosora* showed the highest frond $\delta^{13}C$ among the species under the control conditions.

Closing stomata and/or maintaining photosynthetic machinery during drought stress may cause a higher WUE during drought stress. To analyze the cause of the increase in P_{max}/g_s during drought stress, we compared the relationship between averaged g_s and P_{max}/g_s for the three g_s ranges (Fig. 3). The lowest g_s for *L. thunbergianus*, *D. erythrosora*, and *A. pedatum* were in the same range (Fig. 3, Table 3), whereas the minimum g_s of *P. aquilinum* was more than twofold lower than that of the other species. Conversely, the maximum g_s of *P. aquilinum* was more than twofold higher than the other species.

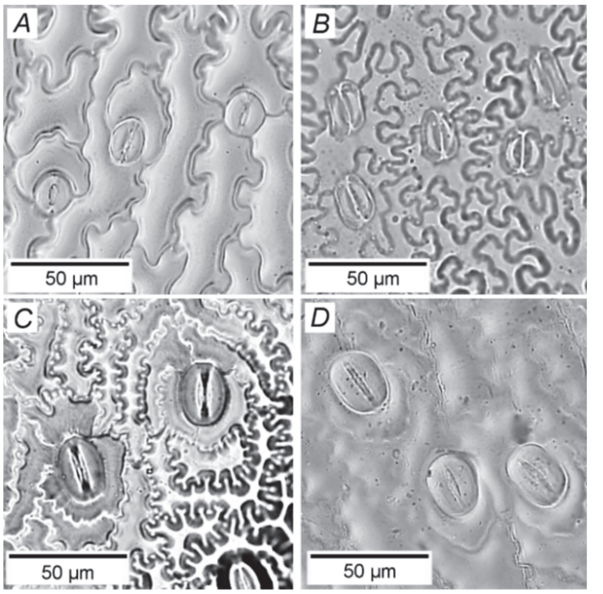

Fig. 1. Stomata on the abaxial side of the fronds of *Adiantum pedatum* (*A*), *Pteridium aquilinum* (*B*), *Dryopteris erythrosora* (*C*), and *Lepisorus thunbergianus* (*D*).

Table 1. Stomatal length, width, density, frond water content (FWC), and frond leaf mass per area (LMA) for four fern species before the onset of drought treatment. Values are means ± SE from four different plants for stomatal properties (*n* = 4), from five stomata from four plants for stomatal length and width (*n* = 20), and from ten different plants for FWC and LMA (*n* = 10). *Different letters* indicate significant differences between species (*ANOVA*, *P*<0.05).

Parameter	*A. pedatum*	*P. aquilinum*	*D. erythrosora*	*L. thunbergianus*
Stomatal length [μm]	31.9 ± 1.1^a	39.3 ± 1.3^b	48.7 ± 1.4^c	49.1 ± 1.6^c
Stomatal width [μm]	26.9 ± 1.4^{ab}	21.8 ± 0.6^a	30.7 ± 2.2^b	38.8 ± 1.6^c
Stomatal density [stomata mm^{-2}]	38.3 ± 1.9^a	117.3 ± 12.5^b	34.9 ± 4.1^a	40.6 ± 2.7^a
FWC [%]	67.0 ± 1.2^a	67.8 ± 2.3^a	61.3 ± 1.8^b	80.3 ± 0.8^c
LMA [g m^{-2}]	28.4 ± 1.6^a	45.4 ± 2.7^a	75.3 ± 6.3^b	99.6 ± 7.7^c

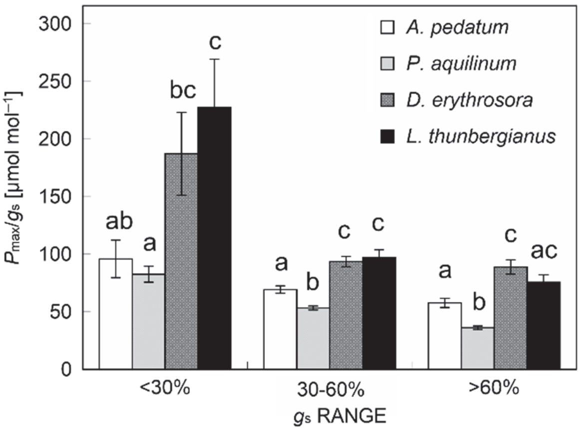

Fig. 2. Photosynthetic water-use efficiency (P_{max}/g_s) of the four fern species for each range of stomatal conductance (g_s), which was calculated as the ratio of the g_s of each species' maximum. *Different letters* indicate significant differences between the species for each g_s range ($P<0.05$, *ANOVA*). When P_{max}/g_s values were compared between g_s ranges, all ferns showed higher P_{max}/g_s at g_s values <30% than those at 30–60% and >60% of the species' maximum ($P<0.05$, *ANOVA*). Values are means ± SE ($n = 4-54$).

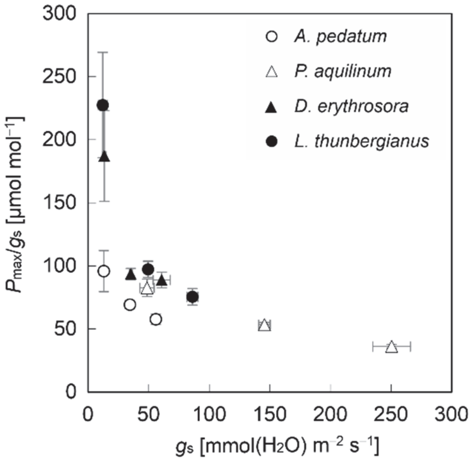

Fig. 3. Relationship between photosynthetic water-use efficiency (P_{max}/g_s) and stomatal conductance (g_s). Data are means ± SE for the g_s ranges of <30%, 30–60%, and >60% of each species' maximum ($n = 4-54$).

Table 2. Average stomatal conductance (g_s) and carbon isotope ratio ($\delta^{13}C$) of fronds for the four fern species in the last week of the drought treatment. Data are means ± SE for control and drought-treated plants, pooled for 2010 and 2011 for g_s ($n = 6-11$) and in 2010 for $\delta^{13}C$ ($n = 4-5$). *Different letters* indicate significant differences between species (*ANOVA*, $P<0.05$). * and ** – statistically significant differences between control and drought-treated plants for each species at $P<0.05$ and $P<0.01$, respectively (*t*-test). n.s. – not statistically significant (*t*-test, $P>0.05$).

	A. pedatum	*P. aquilinum*	*D. erythrosora*	*L. thunbergianus*
Control	0.042 ± 0.004^a	0.108 ± 0.017^b	0.028 ± 0.005^a	0.050 ± 0.004^a
Drought	0.009 ± 0.001^a	0.027 ± 0.014^a	0.014 ± 0.004^a	0.013 ± 0.010^a
Effect of drought	**	**	*	**
Control	-29.4 ± 0.3^b	-29.8 ± 0.2^b	-27.5 ± 0.3^a	-29.3 ± 0.4^b
Drought	-29.1 ± 0.6^b	-29.6 ± 0.3^b	-27.2 ± 0.4^a	-28.6 ± 0.7^{ab}
Effect of drought	n.s.	n.s.	n.s.	n.s.

Table 3. Stomatal conductance (g_s), light-saturated photosynthetic rate (P_{max}), maximum carboxylation rate (V_{cmax}), electron transport rate (J), and Rubisco contents of fronds from four fern species at g_s <30% of species' maximum including data from both control and drought-stress conditions. Rubisco contents were measured in 2011 only. Otherwise, the data from 2010 and 2011 were pooled. Values are mean ± SE ($n = 7-34$). *Different letters* indicate significant differences between species (*ANOVA*, $P<0.05$).

Parameters	*A. pedatum*	*P. aquilinum*	*D. erythrosora*	*L. thunbergianus*
g_s [mmol (H$_2$O) m^{-2} s^{-1}]	13.0 ± 1.5^a	48.6 ± 5.9^b	13.4 ± 1.3^a	12.1 ± 3.6^a
P_{max} [µmol (CO$_2$) m^{-2} s^{-1}]	1.18 ± 0.22^a	3.48 ± 0.37^b	1.70 ± 0.22^a	2.34 ± 0.61^{ab}
V_{cmax} [µmol (CO$_2$) m^{-2} s^{-1}]	14.3 ± 3.2^a	26.9 ± 1.7^b	30.6 ± 2.1^b	59.5 ± 5.7^c
J [µmol (photon) m^{-2} s^{-1}]	25.8 ± 2.7^a	47.5 ± 2.2^b	53.8 ± 2.2^b	92.0 ± 4.2^c
Rubisco [µg cm^{-2}]	49 ± 9^a	140 ± 12^{bc}	98 ± 7^{ab}	173 ± 30^c

P_{max}, V_{cmax}, J, and Rubisco content are related to photosynthetic machinery, and thus may be related to high WUE during drought stress. We compared photosynthetic parameters between species in the g_s range <30%, where drought stress was the highest. P_{max} was higher in *P. aquilinum* than that in *D. erythrosora* and *A. pedatum* (Table 3). *L. thunbergianus* showed the highest V_{cmax} and J among the four species, and the Rubisco content of *L. thunbergianus* was higher than that of *D. erythrosora* and *A. pedatum*.

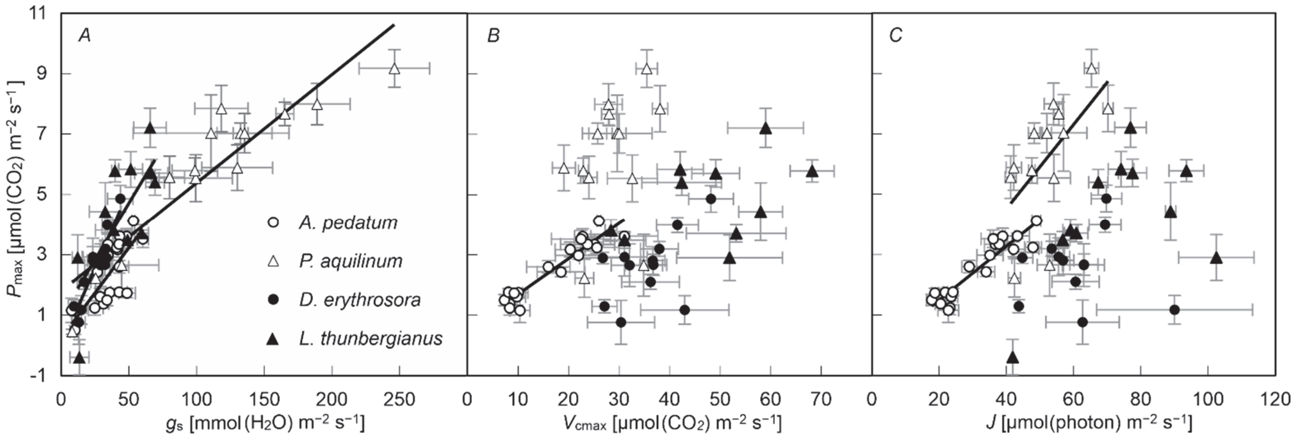

Fig. 4. Correlation between light-saturated photosynthetic rate (P_{max}) and (*A*) stomatal conductance (g_s), (*B*) maximum carboxylation rate (V_{cmax}), and (*C*) electron transport rate (J). Data are means ± SE of 4–6 replicates per species for each gas-exchange measurement for control and drought-treated plants (6–10 measurements in 2010 and 2011). *Solid lines* indicate significant correlations (*Pearson* product-moment correlation, $P<0.05$); $r^2 = 0.84$ for *Pteridium aquilinum*, $r^2 = 0.55$ for *Adiantum pedatum*, $r^2 = 0.89$ for *Dryopteris erythrosora*, and $r^2 = 0.55$ for *Lepisorus thunbergianus* (*A*), $r^2 = 0.90$ for *A. pedatum* (*B*), and $r^2 = 0.35$ for *P. aquilinum*, and $r^2 = 0.83$ for *A. pedatum* (*C*).

There were significant and strong positive correlations between P_{max} and g_s for all fern species (Fig. 4*A*). The correlation between V_{cmax} and P_{max} was significant for *A. pedatum* (Fig. 4*B*). *J* was correlated positively with P_{max} for *A. pedatum* and *P. aquilinum* (Fig. 4*C*). The correlation between Rubisco content and P_{max} was not significant for any of the four species (data not shown).

Drought stress decreased the RFWC of ferns. RFWC decreased from 96 to 66% and 97 to 75% in *P. aquilinum* and *A. pedatum*, respectively (Fig. 5), whereas it decreased from 96 to 66% and from 90 to 30% for *D. erythrosora* and *L. thunbergianus*, respectively. A significant positive correlation was obtained between g_s and RFWC in *A. pedatum* and *L. thunbergianus*.

Discussion

Of the fern species examined, the evergreen epiphytic species *L. thunbergianus* and the evergreen terrestrial species *D. erythrosora* had much higher intrinsic WUE (P_{max}/g_s) than those of the two deciduous species, *A. pedatum* and *P. aquilinum*, during drought stress (g_s <30%, Fig. 2). This result suggested that epiphytic and/or evergreen life-forms evolved high adaptation to water stress conditions among these four fern species. This result partly supports Zhang *et al.* (2009), who found higher P_{max}/g_s in epiphytic compared to terrestrial ferns in a tropical rainforest in China. The increases in P_{max}/g_s during drought, which occurred as a result of adaptive processes, differed between Mediterranean plant species with different life-forms (Flexas *et al.* 2004, Galmés *et al.* 2007), with woody evergreen semi shrubs showing higher P_{max}/g_s, and better adaptation to drought, than herbs and semideciduous shrubs (Gulías *et al.* 2003, Medrano *et al.* 2009). The WUE values of *D. erythrosora* and *L. thunbergianus* at g_s <30% were almost twice those for drought-adapted Mediterranean tree species during drought (Medrano *et al.* 2009), which suggested that *D. erythrosora* and *L. thunbergianus* are highly adaptive to soil drought.

Low g_s and high P_{max} better suited to drought conditions are possible causes of the increase in P_{max}/g_s in response to drought (Flexas and Medrano *et al.* 2002). Low g_s was considered to be an important factor affecting the

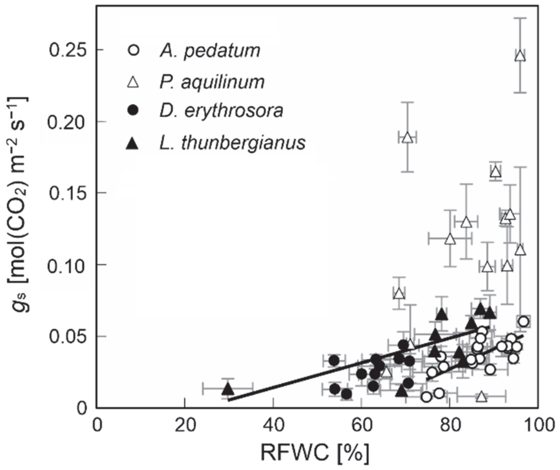

Fig. 5. Correlation between stomatal conductance (g_s) and relative frond water content (RFWC). Data are means ± SE of 4–6 replicates per species for each gas exchange measurement for control and drought-treated plants (6–10 measurements in 2010 and 2011). Solid lines indicate significant correlations (Pearson product-moment correlation, $P<0.05$); $r^2 = 0.55$ for *Adiantum pedatum* and $r^2 = 0.49$ for *Lepisorus thunbergianus*.

higher WUE in *D. erythrosora* and *L. thunbergianus* than that in *P. aquilinum*; the g_s values of *D. erythrosora* and *L. thunbergianus* were only 25–28% of those in *P. aquilinum*, whereas their P_{max} values achieved 49–67% of those in *P. aquilinum* during drought (g_s<30%, Fig. 3, Table 3). These results indicated more sensitive responses of the stomata to drought in *D. erythrosora* and *L. thunbergianus* than in *P. aquilinum*, *e.g.*, the drought avoidance strategy in these two evergreen species. Lower stomatal density in *D. erythrosora* and *L. thunbergianus* than that of *P. aquilinum* (Table 1, Fig. 1*B,C,D*) may also, in part, cause the lower g_s in these evergreen species than in *P. aquilinum*.

On the other hand, the higher P_{max} resulted in higher P_{max}/g_s in *D. erythrosora* and *L. thunbergianus* than that in *A. pedatum* under drought conditions (g_s <30%, Fig. 3, Table 3); the P_{max} values of *D. erythrosora* and *L. thunbergianus* were 144–198% of those in *A. pedatum*, whereas their g_s values were similar to those in *A. pedatum* during drought. The much higher V_{cmax} and J in *D. erythrosora* and *L. thunbergianus* (209–416% of those in *A. pedatum*) indicated better-suited photosynthetic machinery for drought in these evergreen species than that in *A. pedatum* (Table 3), because high V_{cmax} and J are related to high Rubisco and photochemical reaction/Calvin-cycle activities, respectively. The higher Rubisco content in *L. thunbergianus* (353% of that in *A. pedatum*) also resulted in much higher photosynthetic ability than that in *A. pedatum* during drought (Fig. 3, Table 3). These higher photosynthesis abilities in *D. erythrosora* and *L. thunbergianus* than that in *A. pedatum* might be as a result of the thick mesophyll, suggested from their higher LMA values (Table 1).

The g_s values in *P. aquilinum* were the highest among the four ferns in the present study under all water stress conditions (Fig. 3). The high stomatal density of *P. aquilinum* (Table 1, Fig. 1) may contribute to its high g_s; *P. aquilinum* has one of the highest stomatal densities among ferns (Nobel 1978, Hietz and Briones 1998, Tosens *et al.* 2016). Shorter and narrower stomata in *P. aquilinum* (Table 1) suggested that stomatal sizes had a small impact on its high g_s. High g_s and low P_{max}/g_s in *P. aquilinum* under all water-stress conditions (Fig. 2–3) suggested that *P. aquilinum* was not adapted to water-stress conditions, rather it had high adaptation ability to sunny habitats. A high photosynthetic rate was observed for a tree fern *Shaeropteris cooperi* grown at a sunny site, which was related to high g_s (Durand and Goldstein 2001). Under well-watered conditions, *P. aquilinum* has well-developed mesophyll cells, high mesophyll conductance, and high V_{cmax}, J, and P_{max} among fern species, all of which resulted in good photosynthetic performance under high light conditions (Tosens *et al.* 2016). Two tree ferns with good photosynthetic activity, *S. cooperi* (Durand and Goldstein 2001) and *Dicksonia antarctica* (Hunt *et al.* 2002), had also high g_s. In this study, we obtained high g_s with high photosynthetic rate for *P. aquilinum* under mild water-stress conditions (Fig. 4*A*). Additionally, *P. aquilinum* had deep roots and a well-developed vascular system with high hydraulic conductivity (Pittermann *et al.* 2011), which supported its high evaporative demand caused by high g_s in sunny habitats.

The strong positive correlation between P_{max} and g_s for all ferns (Fig. 4*A*) indicated that stomatal regulation strongly contributed to the response of photosynthesis to drought, which was supported Zhang *et al.* (2009) for four tropical ferns. In severe drought conditions, however, physiological impairment, such as decreasing Rubisco content and/or activity, may be possible, which causes a reduction in P_{max} resulting from reductions in V_{cmax} and J (Flexas and Medrano 2002). In this study, V_{cmax} and J of *A. pedatum* were correlated strongly with P_{max} (Fig. 4*B,C*), which suggested that photosynthesis was impaired by drought in *A. pedatum*.

$\delta^{13}C$ of fronds can be used as an index of long-term WUE. Watkins *et al.* (2007) reported higher WUE for a field-grown epiphytic fern than for terrestrial ferns using frond $\delta^{13}C$, which may be due to the difference in water availability in habitats between epiphytic and terrestrial ferns. In the present study, however, $\delta^{13}C$ of the fronds was not affected by drought in any of the species examined (Table 2). This may be due to the relatively short duration of drought treatment in the present study (1–3 weeks); this duration was probably shorter than the time (about one month) needed to induce changes in the integrated isotope ratio of the leaf dry matter (Scartazza *et al.* 1998). Furthermore, the more positive $\delta^{13}C$ of fronds in *D. erythrosora* in well-watered conditions (Table 2) suggested that this species had inherently higher long-term WUE than the other fern species under favorable water availability. This result supported that photosynthetic WUE (P_{max}/g_s) in *D. erythrosora* was relatively high among the four fern species examined in the g_s range of >60% (Fig. 2).

In this study, the epiphytic fern *L. thunbergianus* decreased RFWC significantly during drought treatment (Fig. 5), with its fronds absorbing water vapor from the air during the gas-exchange measurement (data not shown). Additionally, *L. thunbergianus* was able to perform photosynthesis at such a low RFWC (<40%, Fig. 5). These results suggested that the epiphytic fern *L. thunbergianus* can better tolerate low RFWC than the other three terrestrial ferns. This supported the findings of McAdam and Brodribb (2013), who reported that drought-stressed epiphytic ferns were able to tolerate low RFWC (26.7–41.7%) compared to drought-stressed mesophytic terrestrial ferns (>85%). McAdam and Brodribb (2013) also found a large volume of internal leaf water for epiphytic ferns and high tolerance to drought stress. In the present study, the LMA of *L. thunbergianus* was the highest among the four ferns (Table 1), which was strongly affected by its thick fronds, and this may contribute to its large volume of internal leaf water, and thus, may relate to its successful drought adaptation. The genus *Lepisorus* is one of the most recently derived genera among the ferns (Schneider *et al.* 2004, Smith *et al.* 2006), and they are able to retain photosynthetic activity under low RFWC conditions, which is advantageous for their epiphytic life-forms with fluctuating moisture availability, and thus, may contribute to their ecological success in such habitats.

Conclusion: Significant differences in gas-exchange response to drought were obtained between the four fern species examined. The evergreen epiphyte species *L. thunbergianus* and the evergreen terrestrial species *D. erythrosora* showed high P_{max}/g_s during drought stress, which suggested their high adaptation to drought. Small g_s, which was partly affected by low stomatal density, envolved high P_{max}/g_s in *L. thunbergianus* and *D. erythrosora.* The epiphytic fern *L. thunbergianus* showed high V_{cmax} and J during drought stress, which suggested that impairment of its photosynthesis was less severe than that in the other species. On the other hand, the deciduous species *A. pedatum* and *P. aquilinum* changed P_{max}/g_s less effectively during drought stress. High stomatal density was associated with high g_s in *P. aquilinum*, which suggested an adaptive trait for sunny habitats rather than to drought-prone habitats. Life-forms and habitats of ferns involved species-specific adaptations of frond/stomatal morphology and photosynthetic processes that make them successful in water-limiting environments.

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